

Removing the Vertebrate-Specific TBP N Terminus Disrupts Placental β 2m-Dependent Interactions with the Maternal Immune System

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Summary

Mammalian TBP consists of a 180 amino acid core that is common to all eukaryotes, fused to a vertebrate-specific N-terminal domain. We generated mice having a modified *tbp* allele, *tbp* ^{Δ N}, that produces a version of TBP lacking 111 of the 135 vertebrate-specific amino acids. Most *tbp* ^{Δ N/ Δ N} fetuses (>90%) died in mid-gestation from an apparent defect in the placenta. *tbp* ^{Δ N/ Δ N} fetuses could be rescued by supplying them with a wild-type tetraploid placenta. Mutants also could be rescued by rearing them in immunocompromised mothers. In immune-competent mothers, survival of *tbp* ^{Δ N/ Δ N} fetuses increased when fetal/placental β 2m expression was genetically disrupted. These results suggest that the TBP N terminus functions in transcriptional regulation of a placental β 2m-dependent process that favors maternal immunotolerance of pregnancy.

Introduction

Female eutherian (placental) mammals face an odd conundrum. On one hand, mammals have the most advanced defense system against pathogenic insults—the adaptive immune system. This system functions, in part, on the principle of continuous surveillance for presentation of correct “self” antigens by the major histocompatibility complex-I (MHC-I) surface proteins. Surfaces that present foreign antigens are generally attacked and destroyed (Pamer and Cresswell, 1998). On the other hand, the female eutherian’s immune system must tolerate a large and decidedly foreign body, the fetus/placenta (Erlebacher, 2001; Wegmann, 1980).

The mechanisms by which tolerance of the placenta occurs are not yet entirely clear (Erlebacher, 2001; Loke and King, 2000). Although pregnancy sensitizes the mother to paternal antigens (van Kampen et al., 2001), it has only relatively small systemic effects on the mother’s immune competence (Tafari et al., 1995), and priming the mother with paternal or fetal antigens has no effect on pregnancy (Wegmann et al., 1979). Rather, tolerance stems mostly from local effects at the maternal/placental interface (Cross et al., 1994; Rinkenberger et al.,

1997). Most of the effects characterized to date are governed by placental (zygote-derived) gene expression rather than by maternal gene expression. For example, placental trophoblasts produce a number of locally immunosuppressive molecules, including progesterone, indoleamine 2,3-dioxygenase, metal proteases, and inhibitors of complement (Cross et al., 1994; Munn et al., 1998; Van Vlasselaer and Vandeputte, 1984; Xu et al., 2000). Genetic disruption of some of these functions in mice leads to spontaneous abortion (Xu et al., 2000; Yamamoto et al., 1998). Also, trophoblasts at the placental/maternal interface in humans express the placenta-specific nonclassical MHC-I heavy-chain genes HLA-E and HLA-G. Immune-mediated spontaneous abortions often are correlated with failure to express these genes, suggesting that these nonclassical MHC-I genes may play a role in preventing rejection of the placenta (Fuzzi et al., 2002; Hutter et al., 1998; Loke and King, 2000; Pfeiffer et al., 2001; Riteau et al., 2001). Mechanistically, little is known about the gene regulation systems that the placenta uses to evade a maternal rejection response or whether these systems might be useful for protecting other foreign tissue grafts from rejection.

We are interested in understanding how the basal transcription machinery has been specialized for advanced gene regulation in complex multicellular organisms. Whereas *Archaeobacteria* use only a single RNA polymerase to transcribe mRNA, rRNA, and tRNA (Hausner and Thomm, 2001), eukaryotes evolved three specialized RNA polymerases to perform these functions. The TATA binding protein (TBP) is used for promoter recognition during transcription initiation by all of these RNA polymerases in archaea and in eukaryotes. TBP has a 180 amino acid core that is almost perfectly conserved in all species (Hernandez, 1993). That eukaryotic TBP and *Archaeobacteria* TBP evolved from a common ancestor can be inferred by the conserved function, the high degree of amino acid similarity, and the nearly superimposable crystal structures for TBP from each superkingdom (Littlefield et al., 1999). Indeed, between archaea and man, TBP has been more highly conserved by natural selection than even RNA polymerase!

Phylogenetic differences in organism complexity correlate not only to the appearance of new families of structural genes, but also to new families of regulators—transcription factors, mediators, chromatin-modifying proteins, etc.—to control these new genes in their ever more complex and demanding environments. Concomitantly, the role of TBP became more complex. Unlike in archaea, where TBP may function as a single-subunit entity (Qureshi et al., 1997), in lower eukaryotes TBP assembles into three multisubunit factors, SL1, TFIID, and TFIIB, of which TBP is, by mass, only a minor component of each (Hernandez, 1993). SL1 is a part of the complex that directs transcription of the rRNA genes by RNA Pol I; TFIID functions during mRNA transcription initiation by RNA Pol II; and TFIIB functions in the initiation of transcription of tRNAs and some other small RNAs by RNA Pol III. In mammals, TBP interacts with at least one other multiprotein factor, SNAPc, which is in

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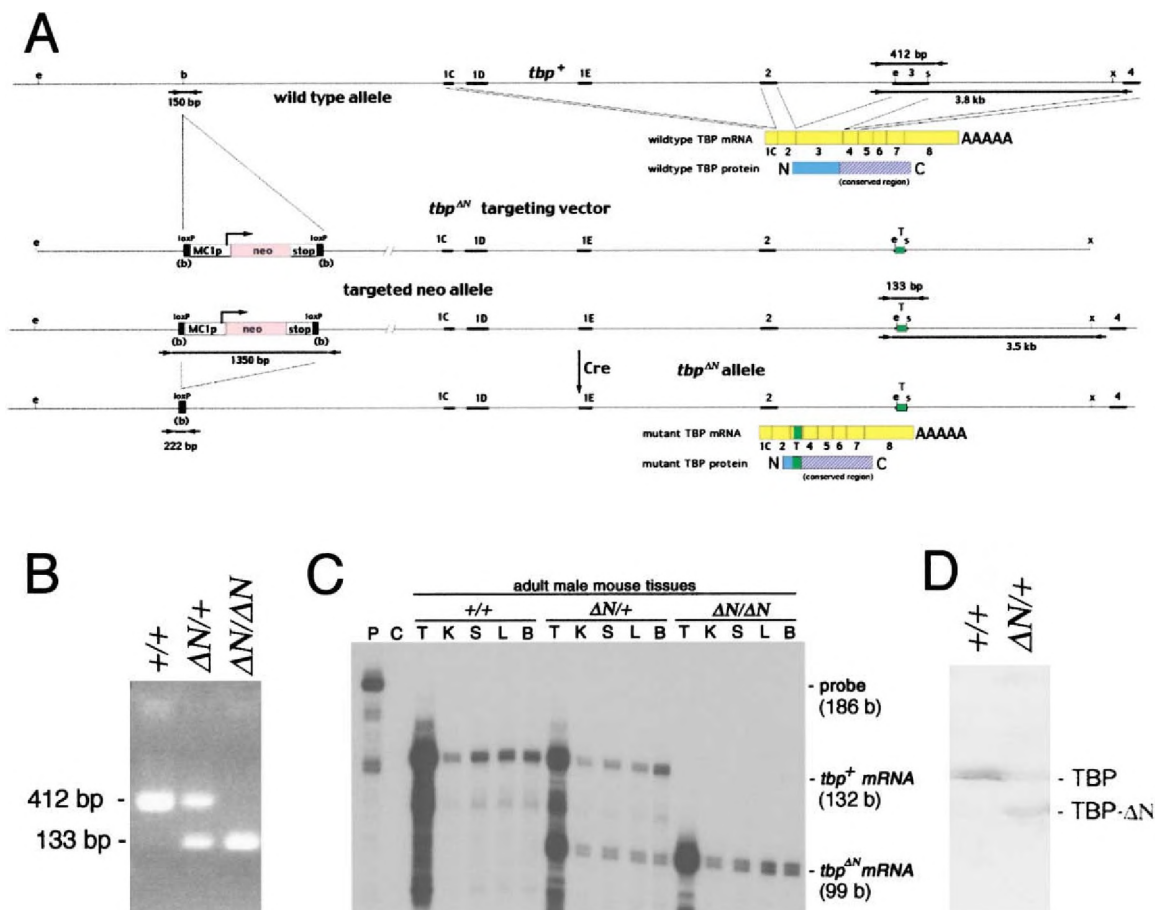


Figure 1. Targeted Mutagenesis of N-Terminal Protein-Coding Sequences of the Mouse *tbp* Gene

(A) Targeting strategy and targeting vector design. The 5' end of the wild-type mouse *tbp* gene (*tbp*⁺) is diagrammed (fine horizontal line on top) indicating 5' exons (thickened regions of line, labeled 1C, 1D, and 1E for alternate promoter/first exons [Ohbayashi et al., 1996; Schmidt et al., 1997], 2, 3, and 4), selected restriction sites (b, Bam HI; e, Eco RI; s, Sac I; x, Xho I), diagnostic PCR primers (arrows), and PCR product sizes (region between arrows, lengths indicated). Below is indicated the predominant splicing pattern (gray broken lines [Schmidt et al., 1997]) that yields the predominant TBP mRNA (yellow) and cognate TBP protein product (blue box). Translation initiates in the second exon and terminates in the eighth exon (Sumita et al., 1993). The N and C termini are indicated. Below is shown the targeting vector design, including the replacement of most of exon 3 with two tandem copies of the FLAG epitope tag (green box). Below this is indicated the targeted *tbp* allele still containing the *loxP*-flanked MC1p-neo gene, with the sizes of diagnostic PCR fragments from the primers shown above on the *tbp*⁺ allele indicated. At the bottom is shown the targeted allele after removal of the *loxP*-flanked MC1p-neo gene by Cre recombinase, with the resultant expressed somatic cell TBP mRNA and TBP protein indicated below.

(B) Genotyping animals using the primer set that spans the ΔN mutation.

(C) Expression of TBP and TBP- ΔN mRNA in mouse cells and tissues. RNase protection assays were performed on 10 μ g of total RNA from the indicated tissues harvested from adult male mice (8- to 12-weeks-old) of the indicated genotypes, supplemented with yeast RNA to 50 μ g (see Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>). Positions of undigested probe, TBP mRNA, and TBP- ΔN mRNA are indicated at right. Abbreviations: P, 1:100 dilution of undigested probe; C, control lane containing probe hybridized to 50 μ g yeast RNA; T, testis; K, kidney; S, spleen; L, liver; and B, brain.

(D) Wild-type and mutant (ΔN) TBP protein expression in adult mouse spleen nuclei (right).

the complex that directs the production of small nuclear RNAs (snRNAs) by either Pol II or Pol III (Hernandez, 1993).

In addition to the 180 amino acid core, TBP acquired a large N-terminal domain in an ancestor to tetrapod vertebrates (Hashimoto et al., 1992). This novel domain represents yet another complexity added to the basal transcription machinery during evolution. We hypothesized that the TBP N terminus might play a role in vertebrate-specific gene regulation. To test this, we designed a mutant allele of the mouse *tbp* gene that we expected to exhibit normal TBP protein expression, but the TBP

protein produced would lack most of the vertebrate-specific TBP N terminus. This mutation did not disrupt basal transcription functions or basal cell physiology. Rather, mouse cells and embryos bearing this mutation were normal by nearly all parameters, and the embryos could, occasionally, develop into adult mice. Importantly, however, most mutants died in midgestation from a placental defect. These mutant fetuses could be rescued by supplying them with a wild-type placenta.

In this paper, we show that most *tbp* ^{$\Delta N/\Delta N$} fetuses survive midgestation in severely immune-compromised mothers. Moreover, in immune-competent mothers,

Table 1. Survival to Weaning

	+/+	$\Delta N/+$	$\Delta N/\Delta N$	(n)
($\Delta N/+$) \times ($\Delta N/+$) matings	141 (100%)	197 (69.9%)	4 (2.8%)	342 ($p < 0.001$) ^a
($\Delta N/\Delta N$) \times ($\Delta N/+$) matings	–	68 (70%) ^b	3 (3.1%) ^b	71 ($p < 0.001$) ^a

Numbers of animals surviving to weaning for 67 litters from heterozygous intercrosses and 11 litters from matings of homozygous mutant males to heterozygous females are indicated. Numbers in parentheses indicate the percent survival of animals of each genotype based on Mendelian ratios of fertilized eggs and 100% survival of wild-type animals, which is valid because litters of pups harvested at 7.5 to 9.5 d.p.c. exhibited Mendelian ratios of all three genotypes (see text).

^a Chi-square test that survival differs from Mendelian genotype ratios, $\alpha = 0.05$.

^b Since matings with homozygous mutant males cannot yield wild-type animals, we assumed that heterozygous pups of these matings, like in the matings of heterozygous parents above, yielded 70% survival. Thus, based on survival of 68 $\Delta N/+$ pups, we calculated that 96 zygotes each of $\Delta N/+$ and $\Delta N/\Delta N$ existed, and homozygous percent survival was derived from this.

tbp ^{$\Delta N/\Delta N$} fetuses are much more likely to survive midgestation if they also lack $\beta 2$ -microglobulin ($\beta 2m$). Our data support the hypothesis that the TBP N terminus is an essential component in a signaling pathway that regulates a placenta-specific $\beta 2m$ -dependent process. This, in turn, may be a part of the mechanism that placentas use to evade a maternal rejection response.

Results

Design and Production of Mice Bearing the *tbp* ^{ΔN} Allele

In previous studies, we had mapped the promoters and first exons of the mouse *tbp* gene (Ohbayashi et al., 1996; Schmidt et al., 1997). This information was used to design a targeting vector that would replace the endogenous gene with a mutant version that was as similar to the original gene as possible except that the protein produced would lack amino acids 25–135 of the vertebrate-specific N terminus (Figure 1A). RNase protection analyses showed that expression of the *tbp* ^{ΔN} allele quantitatively matched that of the wild-type *tbp* allele (Figure 1C). The first 24 amino acids were retained to preserve the relative turnover rate of the mutant protein (Varshavsky, 1997) such that accumulation of the mutant protein would match that of wild-type TBP. Western blotting analyses confirmed that steady-state accumulation of TBP- ΔN protein matched that of wild-type TBP (Figure 1D).

Survival of *tbp* ^{$\Delta N/+$} and *tbp* ^{$\Delta N/\Delta N$} Mice and Embryos

Intercrosses between heterozygous animals yielded 140% as many heterozygous pups and 2.8% as many homozygous mutant pups as they did wild-type pups that survived to weaning (Table 1). This indicated that the *tbp* ^{ΔN} allele was recessive lethal, causing 97% loss of homozygous mutants, and slightly haploid-insufficient, resulting in 30% loss of heterozygotes.

Litters harvested between 7.5 and 9.5 days postfertilization (d.p.f.) exhibited Mendelian ratios of all three genotypes. Between 10.5 and 12.5 d.p.f., numerous embryos were found dying and resorbing, and numbers of recoverable homozygous mutant embryos decreased. Litters harvested between 13.5 and 17.5 d.p.f. contained, on average, 157% as many heterozygous embryos, and 8.7% as many homozygous mutant embryos,

as they did wild-type embryos (Table 2). Thus, the TBP- ΔN mutation caused lethality with 22% penetrance in the heterozygous state and 91% penetrance in the homozygous state between 10.5 and 12.5 days of gestation. No additional loss was detected during gestation; however, there was a 67% loss of the remaining homozygous animals between 17.5 days of gestation and weaning.

Surviving homozygous mutant mice of both genders were healthy and fertile; however, they exhibited no increased incidence of rearing homozygous mutant pups (Table 1). This indicated that their survival did not result from a heritable genetic trait. Moreover, since we have now had several homozygous mutant animals born to homozygous mutant mothers, we can exclude the possibility that the wild-type *tbp* allele of heterozygous mothers rescued the surviving homozygous pups in trans.

Development of *tbp* ^{$\Delta N/+$} and *tbp* ^{$\Delta N/\Delta N$} Mouse Fetuses

Most mutant fetuses died between 10.5 and 12.5 days of gestation; however, all systems, organs, tissues, and cell types appeared to be intact and functioning prior to death (Figures 2A and 2B). The heart was beating, embryonic blood cells appeared normal and were circulating to the most distal small capillaries, and there were no signs of hemorrhage. The only apparent defect in mutant embryos was that they often, but not always, exhibited various degrees of developmental retardation (Figure 2A). Nevertheless, the embryos appeared normal for their “somite-count” stage (Hogan et al., 1994).

Defects in *tbp* ^{$\Delta N/\Delta N$} Placentas

Because we could not find defects in the embryos that accounted for loss of the homozygous mutants, attention was focused on the placenta. If primary failure was due to placental defects, these defects should precede embryonic pathology or death. Embryos and placentas were harvested 10.5 and 11.5 days after mating heterozygous parents. To avoid confusing secondary pathological consequences as putative primary defects, the placentas of embryos that were already dead or resorbing were excluded from analyses. Of the remaining $\Delta N/\Delta N$ placentas, many showed no overt defects, likely because the pathology had not yet progressed to a point that we could detect. However, in about 25% of the placentas, regions could be found in which embryonic and maternal blood were mixing, and clots of maternal

Table 2. Fetal Rescue by Wild-Type Tetraploid Placentas

	+/+	$\Delta N/+$	$\Delta N/\Delta N$	(n)
$(\Delta N/+) \times (\Delta N/+) \text{ matings}$	23 (100%)	36 (78.3%)	2 (8.7%)	61 ($p < 0.001$) ^a
$(\Delta N/+) \times (\Delta N/+) \text{ 2N fetuses on}$ $(+/+) \times (+/+) \text{ 4N placentas}$	7 (100%)	15 (107%)	8 (114%)	30 ($p < 0.001$) ^b

Numbers of fetuses surviving to 15.5 d.p.f. for natural matings between heterozygous animals (7 pregnancies) and for chimeras of diploid morulae of natural matings between heterozygous animals fused to tetraploid morulae from wild-type matings implanted into wild-type surrogate mothers (5 pregnancies). Numbers in parentheses indicate the relative percent survival of animals of each genotype calculated as in Table 1.

^aChi-square analysis of difference from Mendelian 1:2:1 ratio, $\alpha = 0.05$.

^bChi-square analysis of rescue compared to $(\Delta N/+) \times (\Delta N/+) \text{ matings above}$, $\alpha = 0.05$. The ratio of genotypes for the fetuses on wildtype tetraploid placentas (7:15:8) does not differ significantly from a Mendelian 1:2:1 ratio ($\alpha = 0.05$, $p > 0.1$), which is consistent with complete rescue.

blood were abundant (Figure 2C). Also, although trophoblast giant cells are normally phagocytic and occasionally contain hemophagic vesicles, many $\Delta N/\Delta N$ placentas exhibited evidence of far more extensive hemophagocytosis (Figure 2D).

The histopathology suggested that the primary defect of our mutation might be placental. To test this empirically, we used diploid/tetraploid embryo chimeras (Guillemot et al., 1994; Hogan et al., 1994; Nagy et al., 1990) to generate mice where the embryo proper was derived primarily of diploid cells from crosses between $tbp^{\Delta N/+}$ parents, whereas the extraembryonic tissues, including

the placenta, were composed primarily of tetraploid wild-type cells. Results showed Mendelian ratios of fetuses of all three genotypes (Table 2). We conclude that the primary defect of removing the N terminus of TBP in mice is disruption of a situation-specific function, which is required in early postimplantation placentas, but not in fetuses.

Survival of $tbp^{\Delta N/\Delta N}$ Fetuses in Immunocompromised Mothers

Rag1 knockout ($rag1^{-/-}$) mice lack mature B and T cells, leaving them without adaptive immunity (Mombaerts et

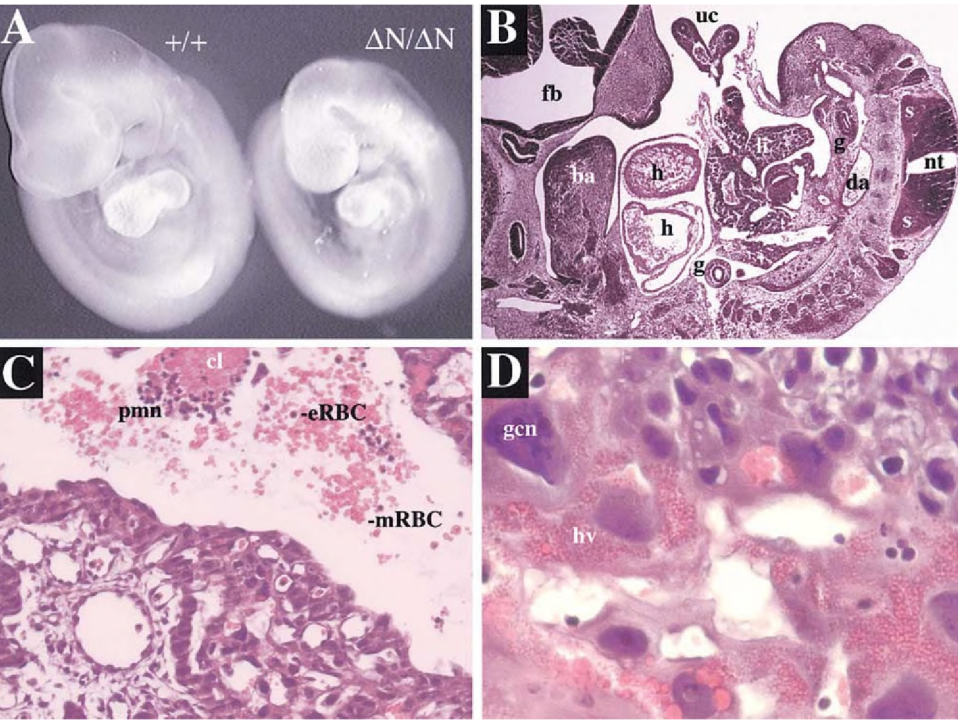


Figure 2. Histopathology of $tbp^{\Delta N/\Delta N}$ Fetuses and Placentas

(A) Wild-type (+/+) and homozygous mutant ($\Delta N/\Delta N$) 10.5 d.p.f. whole fetuses from the same litter.

(B) Histology of 10.5 d.p.f. $\Delta N/\Delta N$ fetus.

(C) Evidence of hemorrhage in placenta, showing a blood sinus with a large clot of maternal blood associated with peripheral polymorphonuclear leukocytes, as well as mixing of maternal and embryonic blood.

(D) Trophoblast giant cells form normally and have normal, large, polyploid nuclei; however, they are engorged with heme-filled vesicles, which suggests that the giant cells in mutant placentas are particularly active in hemophagocytosis.

Abbreviations: ba, brachial arch; cl, clot; da, dorsal aorta; eRBC, embryonic red blood cells; fb, forebrain; g, gut; gcn, giant cell nucleus; h, heart; hv, hemophagic vesicles; li, liver; mRBC, maternal red blood cell; nt, neural tube; s, somite; and uc, umbilical cord.

Table 3. Fetal Rescue Past Midgestation

Fetal Survival as a Function of Maternal <i>rag1</i> Genotype ^a				
Parental Genotypes (Paternal × Maternal)	+/+	ΔN/+	ΔN/ΔN	n (litters [av. size])
<i>tbp</i> ^{ΔN/+} ; <i>rag1</i> ^{+/-} × <i>tbp</i> ^{ΔN/+} ; <i>rag1</i> ^{-/-}	9	20 (111%)	9 (100%)	38 ^b (p < 0.001) ^c (6 [6.3])
<i>tbp</i> ^{ΔN/ΔN} ; <i>rag1</i> ^{+/-} × <i>tbp</i> ^{ΔN/+} ; <i>rag1</i> ^{-/-}	–	15	13 (87%)	28 ^b (5 [5.6])
<i>tbp</i> ^{ΔN/+} ; <i>rag1</i> ^{+/-} × <i>tbp</i> ^{ΔN/+} ; <i>rag1</i> ^{+/-}	23	36 (78%)	2 (8.7%)	61 ^d (9 [6.8])
Fetal Survival as a Function of Fetal <i>β2m</i> Genotype ^e				
Fetal Genotype	+/+	ΔN/+	ΔN/ΔN	n
<i>β2m</i> ^{+/-}	25	42 (84%)	2 (8%)	69
<i>β2m</i> ^{-/-}	29	55 (95%)	14 (48%)	98 (p < 0.001) ^f

^a For matings with *tbp*^{ΔN/+} fathers, we assumed 100% survival of *tbp*^{+/-} fetuses to estimate percent survival; for matings with *tbp*^{ΔN/ΔN} fathers, we assumed 100% survival of heterozygous fetuses.

^b All fetuses *rag1*^{-/-}.

^c Chi-square test that genotype ratio differs significantly from *tbp*^{ΔN/+}; *rag1*^{+/-} × *tbp*^{ΔN/+}; *rag1*^{+/-} mating shown below, as a measure of rescue, α = 0.05.

^d All fetuses *rag1*^{+/-}.

^e To ensure that all animals had an equivalent immune-competent maternal environment, all litters were from *tbp*^{ΔN/+}; *β2m*^{+/-} females mated with males which were *tbp*^{ΔN/+}; *β2m*^{+/-}; *tbp*^{ΔN/+}; *β2m*^{-/-}; or *tbp*^{ΔN/+}; *β2m*^{-/-}. Fetuses were genotyped for both *tbp* and for *β2m*, and data are segregated based on the fetal *β2m* genotype.

^f Chi-square test that *tbp* genotype ratios of *β2m*^{-/-} fetuses are significantly different from those of *β2m*^{+/-} fetuses, α = 0.05.

al., 1992). We asked whether the *rag1*^{-/-} mutation in mothers could genetically complement the *tbp*^{ΔN/ΔN} condition in the fetus/placenta. Mice bearing the *tbp*^{ΔN} mutation were crossed into the Rag1 knockout line to obtain females that were *tbp*^{ΔN/+}; *rag1*^{-/-}. These females were used in timed matings with *tbp*^{ΔN/+}; *rag1*^{+/-} males or with *tbp*^{ΔN/ΔN}; *rag1*^{+/-} males such that all zygotes would be *rag1*^{+/-}. Results showed that roughly 92% of the *tbp*^{ΔN/ΔN} fetuses in these matings survived the midgestational block (Table 3). Similar results were found when the *tbp*^{ΔN} mutation was bred into SCID mice (see Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>). Since survival could be achieved by altering the maternal environment, it is unlikely that *tbp*^{ΔN/ΔN} placentas were intrinsically unable to support a fetus, but rather they interacted inappropriately with immune-competent mothers. Thus, death of *tbp*^{ΔN/ΔN} fetuses in immune-competent mothers resulted, at least in part, from a placental defect that led to maternal rejection.

Genetic Rescue of *tbp*^{ΔN/ΔN} Fetuses by Disruption of Fetal/Placental *β2m* Expression

The role of the maternal immune system in failure of *tbp*^{ΔN/ΔN} fetuses led us to hypothesize that the defect involved inappropriate antigen presentation. Most MHC-I/MHC-II-like heavy chains require the common light chain, *β2m*, for assembly and subsequent surface presentation (Margulies, 1999; Pamer and Cresswell, 1998). Thus, if rejection of the *tbp*^{ΔN/ΔN} fetuses involved MHC-I or MHC-II-like molecules, then the defect should be complemented by the *β2m* knockout (Koller et al., 1990). The *tbp*^{ΔN} mutation was crossed into the *β2m* knockout line to obtain *tbp*^{ΔN/+}; *β2m*^{+/-} females. These females were bred with males that were *tbp*^{ΔN/+}; *β2m*^{+/-}, *tbp*^{ΔN/+}; *β2m*^{-/-}, or *tbp*^{ΔN/ΔN}; *β2m*^{-/-}, fetuses were harvested after midgestation, and genotypes were determined for both *tbp* and *β2m*. Whereas only 8% of the *tbp*^{ΔN/ΔN} fetuses that were *β2m*^{+/-} survived, 6-fold more (48%) of the *tbp*^{ΔN/ΔN} fetuses that were *β2m*^{-/-} survived (Table 3). We conclude that the defect in *tbp*^{ΔN/ΔN} placen-

tas involves failure of a *β2m*-dependent process that can be genetically rescued by obliterating placental *β2m* expression.

Rejection of *tbp*^{ΔN/ΔN} Placentas Is a Local Response

We wished to determine whether those homozygous embryos that survived in immune-competent mothers did so because particular mothers were more tolerant of the mutants (i.e., maternal determinants), or because particular *tbp*^{ΔN/ΔN} placentas failed to “trigger” the rejection response (i.e., placental determinants). We hypothesized that if maternal determinants allowed tolerance of *tbp*^{ΔN/ΔN} placentas, then in those pregnancies, all *tbp*^{ΔN/ΔN} fetuses should survive. Conversely, if placental determinants were responsible for survival, then it should be possible to find surviving *tbp*^{ΔN/ΔN} fetuses in pregnancies in which other *tbp*^{ΔN/ΔN} fetus were being rejected. To address this, we set up *tbp*^{ΔN/+} × *tbp*^{ΔN/+} matings and examined the genotype ratios and numbers of resorbing fetuses in mothers in which one or more *tbp*^{ΔN/ΔN} fetuses survived past midgestation. From six pregnancies we found only one in which no fetuses were being resorbed, only one litter in which more than one *tbp*^{ΔN/ΔN} fetus survived midgestation, and, overall, sub-Mendelian representation of *tbp*^{ΔN/ΔN} fetuses (Table 4). Moreover, our colony has had two successful pregnancies from matings of *tbp*^{ΔN/ΔN} males with *tbp*^{ΔN/ΔN} females, in which all zygotes would be *tbp*^{ΔN/ΔN} (see Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>). These two pregnancies led to only three surviving pups, although in matings with wild-type animals, both *tbp*^{ΔN/ΔN} males and *tbp*^{ΔN/ΔN} females showed normal litter sizes (data not shown), indicating sperm production, egg production, and fertilization were unaffected. Thus, it is almost certain that, despite yielding three live pups, multiple homozygous fetus/placentas were rejected in these two pregnancies. The results strongly suggest that individual *tbp*^{ΔN/ΔN} fetuses can survive in litters in which other *tbp*^{ΔN/ΔN} fetuses are being resorbed. There-

Table 4. Resorbing Fetuses from $tbp^{\Delta N/+} \times tbp^{\Delta N/+}$ Matings in Midgestation Litters that Contained Live $tbp^{\Delta N/\Delta N}$ Fetuses

Litter	Genotype Ratios of Live Fetuses ^a (+/+: ΔN /+: ΔN / ΔN)	Number of Resorbing Fetuses in Litter ^b
A	4:2:1	4
B	3:3:2	1
C	2:4:1	4
D	1:5:1	0
E	0:2:1	2
F	2:4:1	1
Sum of live fetus genotypes	12:20:7	
Total fetuses (live + resorbing)	51 fetuses	
Estimated zygote genotypes ^c	13:25:13	
Predicted resorbing genotypes ^d	1:5:6	

^aOnly litters containing $tbp^{\Delta N/\Delta N}$ fetuses are included, thus inflating representation of $tbp^{\Delta N/\Delta N}$ fetuses as compared to Table 1. C57Bl/6 background, no rescuing mutations.

^bResorbing fetuses could not be genotyped.

^cEstimate based on best fit to Mendelian ratio.

^dPrediction based on best mathematical fit for the equation: resorbing genotypes = estimated zygote genotypes – live fetus genotypes.

fore, survival of individual $tbp^{\Delta N/\Delta N}$ fetuses is a “trait” of those particular fetuses and not of the mother. Interestingly, since $tbp^{\Delta N/\Delta N}$ fetuses from $tbp^{\Delta N/\Delta N}$ parents are no more likely to survive than are $tbp^{\Delta N/\Delta N}$ fetuses from $tbp^{\Delta N/+}$ parents (see Supplemental Data), this trait is not heritable (i.e., not germline genetic). We conclude that the rejection of $tbp^{\Delta N/\Delta N}$ fetuses requires both a genetic component that is dependent on the tbp mutation and a nonheritable “triggering component” (see Discussion).

Rejection of $tbp^{\Delta N/\Delta N}$ Fetuses Does Not Involve a Memory Response

If rejection of the $tbp^{\Delta N/\Delta N}$ fetuses were a classical graft rejection response, it should have a memory component, such that once a mother had rejected a mutant fetus, mutants in subsequent pregnancies would be more aggressively rejected. Were this the case, all surviving homozygous animals should have been born to mothers that had not previously rejected fetuses. To test this prediction, we examined the maternal history of the $tbp^{\Delta N/\Delta N}$ adults that have survived from natural matings in our colony in the absence of rescuing mutations. Results showed no significant correlation between homozygote survival and maternal history (see Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>). Only 10 of the 17 pups were from a mother's first litter. In one exceptional case, a heterozygous mother had four litters, one with a heterozygous mate and three with homozygous mates. Of the 21 pups weaned, two were homozygous mutants—one in her first litter and one in her fourth—and 17 were heterozygotes. Based on our calculation of 70% survival of heterozygotes in these matings (Table 1), during these four pregnancies, she lost roughly 10 $tbp^{\Delta N/\Delta N}$ and 7 $tbp^{\Delta N/+}$ fetuses, but she still tolerated a $tbp^{\Delta N/\Delta N}$ fetus in the fourth litter. We conclude that there is no detectable memory component to the maternal rejection response on $tbp^{\Delta N/\Delta N}$ fetuses in syngeneic matings.

Discussion

We have generated a line of mice in which the endogenous tbp gene was replaced by a version that produces

a protein lacking the vertebrate-specific N terminus. Although rare, homozygous adults of both genders are healthy and fertile, indicating that this domain of TBP is not required for most vital functions. Nevertheless, >90% of the homozygous mutants died in midgestation from a placental defect. The data indicated that a second crisis occurred between late gestation (17.5 d.p.f.) and weaning, which eliminated about 67% of the remaining mutants. Because so few mutants survived the midgestational crisis in normal matings, we have not yet initiated a study of this later crisis point. However, preliminary data from complementation analyses in immunocompromised mothers suggest that this second crisis is independent of the mother's immune status. The increased midgestational survival obtained by using mothers carrying loss-of-function mutations in *scid* or *rag1* might provide a system in which we can begin to investigate the cause of this second crisis.

Evolution of the Basal Transcription Machinery

The chemistry of DNA-dependent RNA polymerization, or transcription, has changed little during evolution; however, the enzymatic machinery that catalyzes this process, the basal transcription machinery, shows enormous differences (Figure 3A). As life forms evolved greater complexity, new genes and gene families arose to carry out novel tasks. Concomitantly, new regulators were required to restrict transcription of these new genes to specific situations. We posit that the basal transcription machinery coevolved with these target genes and regulators to facilitate appropriate “situation-specific” regulation of these novel genetic pathways. Just like an old computer may lack the ports needed to communicate with new accessories, the basal transcription machinery likely needed to acquire new “communication ports” to participate in advanced gene regulation. We hypothesize that many of the embellishments added to the basal transcription machinery during evolution function as specific signaling ports. To test this hypothesis, we removed one such embellishment—the TBP N terminus.

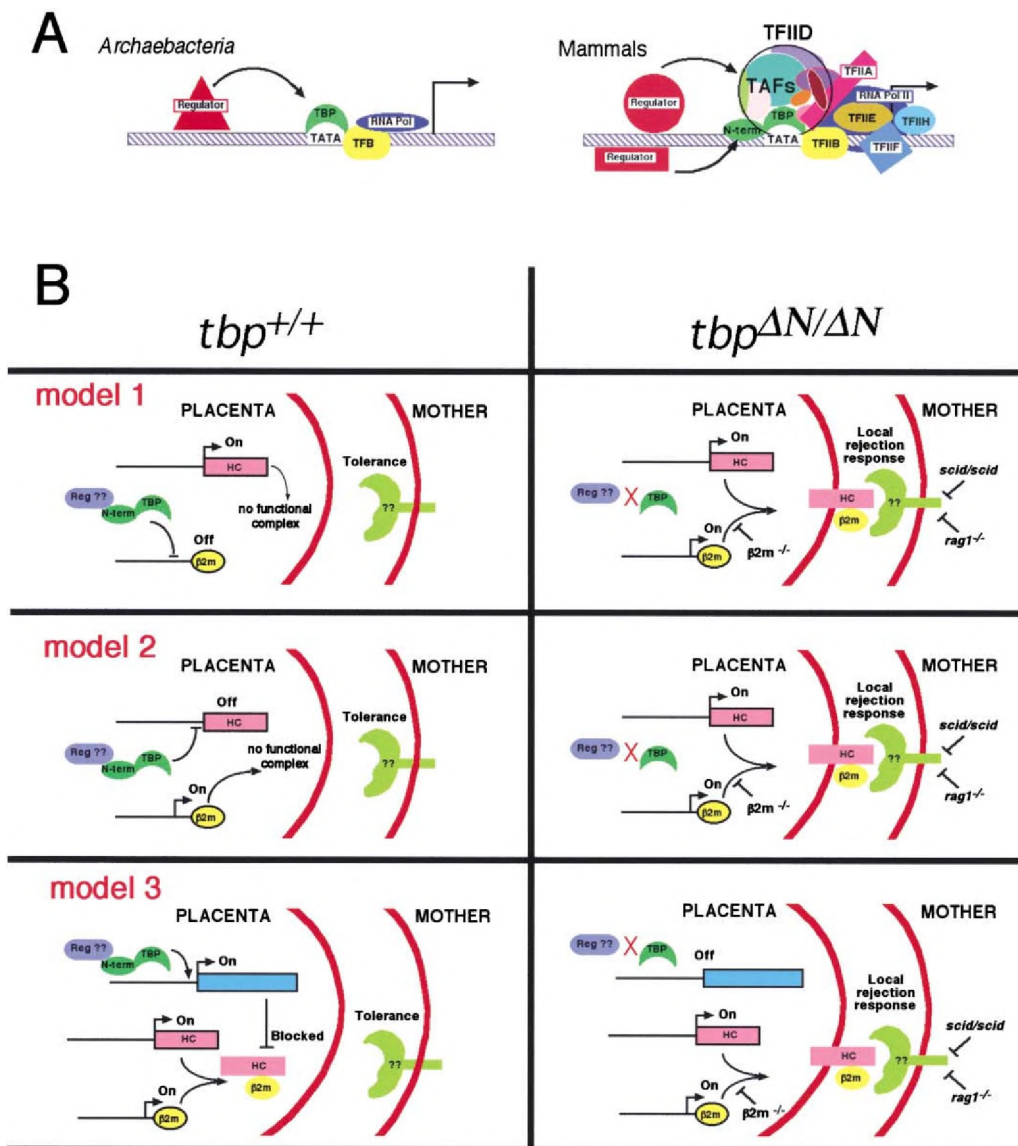


Figure 3. Models of the Function of the N Terminus of TBP

(A) Evolution's embellishments on the basal transcription machinery. Model depicts the basal transcription machinery interacting with transcriptional regulators on promoters in archaea and in mammals. The diverse embellishments on the mammalian transcription machinery are posited to provide novel interaction surfaces that coevolved with the novel transcriptional regulators for advanced situation-specific gene regulation. (B) Three models of TBP- ΔN -mediated placental gene expression in the maternal/placental interaction. In models 1 and 2, signaling through the TBP N terminus represses expression of $\beta 2m$ -dependent antigen presentation, either by directly repressing expression of $\beta 2m$ (yellow, model 1) or expression of a MHC-I or MHC-I-like heavy chain (in pink, model 2). The $\beta 2m$ -dependent antigen presentation is then detected by an as yet unidentified maternal receptor (light green). In these two models, the regulator (labeled "Reg," in purple) would be a repressor. The molecular interaction disrupted by the TBP- ΔN mutation is indicated by a large red "X." Conversely, in model 3, the regulator may be an activator, and signaling through the TBP N terminus may induce expression of another target gene (labeled "TG," in blue), which attenuates, modifies, or blocks placental $\beta 2m$ -dependent antigen presentation. This could occur intracellularly, as depicted, or extracellularly, by masking the $\beta 2m$ -dependent antigen from the maternal receptor. Points in the process that are affected by the rescuing mutations are indicated as " $\beta 2m$ ^{-/-}," "*scid/scid*," and "*rag1*^{-/-}."

The N Terminus of TBP as a "Covalent TAF"

In mammals, TBP functions at the core of the multiprotein factor TFIID for gene expression (Dymlacht et al., 1991; Takada et al., 1992); however, in *Archaeobacteria*, TBP likely acts alone in its homologous role (Qureshi et al., 1997). The other components of mammalian TFIID are proteins known as TBP-associated factors of TFIID, or TAF_{II}s (Hernandez, 1993). Several TAF_{II}s have been

shown to interact directly with transcription factors or mediators, whereas others exhibit enzymatic properties that may only function in specific situations (Albright and Tjian, 2000). We suspect that many TAF_{II}s, like the N terminus of TBP, arose during evolution as ports or accessories for advanced genetic processes. Since more primitive organisms thrive without these embellishments, one can predict that mice bearing mutations

in late-evolving TAF_{II}s will generally result in situation-specific defects (see below), as reported here for the TBP N terminus. Indeed, we consider the N terminus of TBP itself to be a TAF, which is covalently linked to TBP as a fusion protein.

Recently, several tissue-specific components of TFIID or TFIID-related factors have been genetically disrupted in metazoan animals. A screen for male-sterile mutants in *Drosophila* revealed one locus, entitled *cannonball*, that affects spermatogenesis. Positional mapping and functional analysis indicates that this locus encodes a testis-specific TAF_{II} (Hiller et al., 2001). The gene encoding the one known mammalian TBP family member, TRF-2, which is most predominantly expressed in testis, has been knocked out in mice and this mutation, too, leads to male sterility (Zhang et al., 2001). Finally, a mouse tissue-specific TAF_{II}, TAF_{II}105, has been knocked out, leading to a defect in oogenesis and female sterility (Freiman et al., 2001). All three of these mutations are in TFIID components that likely evolved in metazoan animals, and these mutations, like the *tbp*^{ΔN} mutation, have little or no effect on basal functions that are shared with more primitive life forms. In all of these examples, one might posit that the mutation eliminated a situation-specific signaling port that is only required for highly specialized regulation pathways. Unlike the others, however, the *tbp*^{ΔN} mutation is the first to disrupt a ubiquitously expressed component of TFIID.

Placental Consequences of the *tbp*^{ΔN} Mutation

Previous reports of mutant mice exhibiting placental defects can be segregated into those that cause autonomous defects in the placenta and those that cause defects in how the placenta interacts with the mother. For example, null mutations in the transcription factors Mash 2, Ets2, or I-mfa, all of which are required for development or function of placental trophoblasts, lead to autonomous defects that have been rescued by altering the placenta (i.e., providing an alternate source of trophoblast cells), but not by altering the maternal environment (Guillemot et al., 1994; Kraut et al., 1998; Rossant et al., 1998; Tanaka et al., 1997; Yamamoto et al., 1998). Conversely, disruption of a complement inhibitor, Crry, leads to an interaction defect between the placenta and the maternal immune system that can be rescued by rearing the fetus in mothers incapable of mounting a normal complement reaction (Xu et al., 2000).

Genetic complementation showed that the defect in *tbp*^{ΔN/ΔN} placentas was an interaction defect rather than an autonomous defect. In this study, genetic rescue of mutant fetuses was achieved by two completely different approaches. In the first, genetic alteration of the mother, specifically disrupting V(D)J recombination, provided a maternal environment that allowed survival of most mutant fetuses. In the second, second-site genetic modification of the zygotic genome, specifically interfering with their ability to present MHC-I/MHC-I-like antigens, caused a 6-fold increase in survival in immune-competent mothers.

The N Terminus of TBP Is Not Required for Vital Fetal or Adult Functions

Prior to resorption, many mutant fetuses showed signs of developmental retardation; however, for several rea-

sons we believe that this was a secondary consequence of the placental defect. First, the extent of placental histolysis roughly correlated with the degree of developmental retardation (data not shown). Second, mutant fetuses having wild-type tetraploid placentas survived (Table 2) and were not developmentally retarded (data not shown). Finally, most mutant fetuses survive in mothers incapable of mounting a rejection response, and surviving adults are healthy and fertile. The simplest explanation for the developmental retardation of $\Delta N/\Delta N$ fetuses is that placental failure compromises development of the fetus by causing inefficient nutrient, gas, or waste exchange with the mother or by otherwise failing to fully support the developing fetus.

The N Terminus of TBP Favors Survival of Postimplantation Placentas, But Its Function Is Not Likely "Placenta Specific"

The *tbp*^{ΔN} allele was designed on the premise that this protein domain serves as a communication port for transcriptional regulation of a process found only in species that possess this sequence. Six years ago, when we designed and produced these mice, public sequence data indicated that tetrapod vertebrates contained the TBP N terminus, but lower metazoans, including insects and echinoderms, did not. Therefore, we expected to find a defect in an "advanced vertebrate-specific characteristic," such as body form, lungs, adaptations for terrestrial life, etc. The placental defect reported here was not expected because the TBP N terminus exists in amphibia, reptiles, and birds, which generally lack placentas. We have recently cloned TBP cDNAs from species between echinoderms and amphibia, including amphioxus, lamprey, shark, and bony fish (A.A.B., K. Daughenbaugh, M.R.C., and E.E.S., unpublished). These sequences indicate that this region of TBP arose and was conserved by natural selection for more than three hundred million years before the appearance of placental mammals. Why, then, do our mice die from a placental defect?

In this paper, we present genetic evidence that removing the TBP N terminus disrupts a $\beta 2m$ -dependent process that the placenta uses to evade a maternal rejection response. Evidence of $\beta 2m$ -dependent processes, most notably MHC-I presentation, has been found in nearly all vertebrate species, but is absent from other life forms (Du Pasquier and Flajnik, 1999). In other experiments, we have found that the TBP N terminus may have coevolved with the MHC system, which suggests that it might serve as a signaling port for regulating a subset of MHC activities (manuscript in preparation). We propose that the mammalian placenta "coopted" this port, its regulators, and its target genes to create a genetic mechanism for attenuating or evading the maternal immune system in this very special situation. The data suggest that our mice die because, in mammals, this placental role is the first vital function of the pathway. We can rear adult homozygous mutants because, under our care conditions, other functions of this pathway, including those upon which natural selection acted for over three hundred million years preceding the appearance of eutherian mammals, are not vital.

The TBP N Terminus and MHC

Nearly all nucleated cells in the body express MHC-I, which plays a key role in host defense against viruses (Pamer and Cresswell, 1998). Above, we proposed that the TBP N terminus might be considered a TAF that is linked as a fusion protein to the TBP C terminus, or in other words, a "covalent TAF." Of the many TAFs in mammals, why would natural selection have determined that this one, in particular, should be fused to TBP C-terminal core? The answer might lie in MHC-I function. MHC-I heavy-chain genes, $\beta 2m$, and *tbp* (including the vertebrate-specific N terminus) are among the only "vertebrate-specific genes" that, rather than being expressed in a tissue-specific fashion, are expressed in most cells of the body. Natural selection may have favored fusion of this novel domain to the TBP C-terminal core because it would ensure that all cells will correctly express MHC-I. Conversely, if the TBP N terminus were a separate polypeptide not fused to TBP, intracellular pathogens may have more easily evaded MHC-I-mediated host defenses by interfering with expression of this TAF while preserving host cell TBP expression. We are currently beginning studies to determine whether *tbp* ^{$\Delta N/\Delta N$} adults exhibit defects in $\beta 2m$ -dependent processes, including their susceptibility to intracellular pathogens.

Our genetic data are consistent with several mechanistic models (Figure 3B). In the first and simplest, signaling through the TBP N terminus might play a direct role in downregulating $\beta 2m$ expression in key placental cells. In a second model, signaling through the TBP N terminus might play a role in downregulating placental expression of a MHC-I or a MHC-I-like heavy chain. Finally, signaling through the TBP N terminus might play a role in upregulating a placental gene that functions to mask or block MHC-I or MHC-I-like molecule presentation by the placenta. Ongoing work is aimed at testing these three models.

Maternal Components of the Rejection Response

Generally, when MHC molecules are implicated in rejection, haplotype differences play a role in the response (Auchincloss et al., 1999). The *tbp* ^{ΔN} mutation was generated on strain 129X1 and was extensively backcrossed to strain C57Bl/6, both of which are haplotype b/b. Moreover, the *tbp* gene, and thus the *tbp* ^{ΔN} allele, is tightly linked to the MHC complex on chromosome 17 (*tbp* and the MHC also share a common chromosome in humans, but with a more distant linkage). As such, all mice presented in this paper were syngeneic haplotype b/b. Survival to weaning had no gender bias (see Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>), excluding a role for sex-linked antigens. In outbreeding experiments, there was no strain affects in relationship to survival of *tbp* ^{$\Delta N/\Delta N$} fetuses (see Supplemental Data). However, even in these studies, due to the tight linkage of *tbp* and the MHC complex, nearly all *tbp* ^{$\Delta N/\Delta N$} fetus/placentas were haplotype b/b, and all mothers, being *tbp* ^{$\Delta N/+$} , carried at least one haplotype b MHC complex. Therefore, none of the *tbp* ^{$\Delta N/\Delta N$} fetus/placentas in this study contained any autosomal loci that were also not present, at least in heterozygous form, in the mother. Antigenic differences between the

mother and the fetus/placenta, if any existed, resulted not from differential gene possession, but rather from differential gene expression.

Most models of immune rejection of pregnancies, for example in matings between CBA and C57Bl/6 mice (Munn et al., 1998; Tafuri et al., 1995), are based on conditions in which, like in graft rejection, heterogeneity at the MHC loci can play a major role in the rejection response. At least one previous study, however, shows that chimeric misexpression of MHC-I transgenes that match the mother's haplotype can lead to placental failure (Jaffe et al., 1992). Because failure occurred in situations where the transgene and the mother were syngeneic, the authors concluded that the mechanisms of placental failure were not likely immune mediated (Jaffe et al., 1992). By contrast in our study, since *tbp* ^{$\Delta N/\Delta N$} fetus/placentas could be rescued by rearing them in *rag1* ^{$-/-$} or *scid/scid* mothers, maternal immune components very likely participated in the rejection response, and a haplotype-independent immune response should likely be invoked in models to explain our results. One intriguing hypothesis that is consistent with many of our observations is the "danger" model of immunity, in which immune responses can be triggered by endogenous alarm or "distressed cell" signals rather than by nonself antigen (Matzinger, 2002). Since *tbp* ^{$\Delta N/\Delta N$} cells, fetuses, and adults showed no evidence of physiological perturbations, however, cellular distress in *tbp* ^{$\Delta N/\Delta N$} mice would likely have to be highly localized to the placenta. Alternatively, one might invoke a model based on haplotype-independent antigens.

It is interesting to consider what haplotype-independent antigens might participate in rejection of the mutant placentas. Some endogenous genes encode potentially autoantigenic molecules. Examples include cancer antigens, immune-sequestered molecules (e.g., nuclear complexes), and developmentally late appearing molecules (e.g., sperm-specific antigens) (Hall et al., 1994; Schreiber, 1999; Shevach, 1999). Some fetal/placental proteins may also be antigenic due to their normal tissue/stage-restricted expression (Wegmann et al., 1979). Moreover, some nonclassical MHC-I genes are likely expressed only in the placenta, whereas other MHC-I molecules might be strictly repressed in normal placenta (Fuzzi et al., 2002; Hunziker and Wegmann, 1986; Hutter et al., 1998; Jaffe et al., 1991; Loke and King, 2000; Pfeiffer et al., 2001; Riteau et al., 2001). Misregulation of these genes could be responsible for failure of the mutant placentas. Finally, other possibilities should be considered. For example, at least one endogenous retrovirus is known to encode a protein, syncytin, that is required for development of placental giant cells (Mi et al., 2000). One might consider the possibility that the TBP N terminus plays a role in activating or repressing activation of certain germline retroviruses or retroviral genes in the placenta.

Besides occurring in syngeneic conditions, other results reported here suggest that rejection of *tbp* ^{$\Delta N/\Delta N$} fetuses by immune-competent mothers is not likely a classical rejection response. First, we see no evidence of memory. Second, rejection of one placenta does not necessarily compromise other mutant placentas in that pregnancy, indicating that the maternal response is localized to individual placentas and suggesting this re-

sponse has no systemic component. Finally, whereas genetic disruption of $\beta 2m$ rescued most $tbp^{\Delta N/\Delta N}$ fetuses, $\beta 2m^{-/-}$ tissue grafts are vigorously rejected in a classical rejection response (Li and Faustman, 1993).

$rag1^{-/-}$ mice have severely compromised adaptive immunity. On the other hand, these mice exhibit relatively normal levels of innate immune system components, including NK cells, macrophages, and complement. Because $tbp^{\Delta N/\Delta N}$ fetuses were partially rescued by rearing them in $rag1^{-/-}$ mothers, the maternal adaptive immune system likely played a key role in the failure of $tbp^{\Delta N/\Delta N}$ fetuses in immune-competent mothers. However, because the adaptive and innate immune systems modulate the activities of each other, it is likely that other maternal factors contribute to the rejection response. One must consider the possibility that failure of the mutant placentas involves an altered inflammatory response, in which the mother's adaptive immune system could play a regulatory role, an effector role, or both. Numerous recent studies have implicated both adaptive and innate immunity in placental tolerance/rejection (reviewed in Erlebacher, 2001), and we consider it likely that aspects of both innate and adaptive immunity contribute to failure of $tbp^{\Delta N/\Delta N}$ fetuses.

It is uncertain which components of the adaptive immune system are functioning in placental rejection. Since the effect was highly localized and lacked a detectable memory component, it is unlikely that classical humoral immunity played a pivotal role in the response. Several recently characterized placental immune cells fit our data as candidate effector cells that could cause rejection of $tbp^{\Delta N/\Delta N}$ fetuses but would not develop in SCID or $rag1^{-/-}$ mice. One is maternal-derived placental $V\alpha 14$ NKT cells, which respond to an as yet unidentified non-CD1 MHC-I-like $\beta 2m$ -dependent molecule on the placenta (Dang and Heyborne, 2001). These cells are distinct from NKT cells in other maternal organs, which suggests that they develop de novo in the placenta (Dang and Heyborne, 2001). Another candidate is maternal $V\alpha 11$ T cells, which develop in the placenta and may be involved in natural abortion (Yamasaki et al., 2001). If the immune effector cells involved in rejecting $tbp^{\Delta N/\Delta N}$ fetuses developed de novo in each placenta, it may explain why survival differs for individual $tbp^{\Delta N/\Delta N}$ fetuses/placentas within a single litter (Table 4). Moreover, if these cells were lost with the placenta at birth, it may explain the absence of a memory component to this rejection response.

TBP N Terminus-Independent Components of Placental Failure

Removal of the TBP N terminus genetically predisposed our mice to placental rejection; however, rejection was only $\sim 91\%$ penetrant, indicating that an additional "triggering" component participated in determining whether mutant placentas were rejected. Our results indicate that this component is placental, not maternal, and that this component is not heritable. Based on these observations, it was unlikely that germline-genetic properties determined whether mutant placentas were rejected. Instead, we suspect that the triggering component is stochastic.

It is interesting to speculate about this stochastic pla-

cental component. One simple model might be that the triggering event is placental hemorrhage. If spontaneous hemorrhage occurs naturally and then heals in $\sim 90\%$ of placentas, and this hemorrhage exposes the TBP- ΔN -dependent defect to the maternal immune system, it might account for survival of those few $tbp^{\Delta N/\Delta N}$ fetuses through midgestation. The observation that nongenetic components can override the genetic predisposition of $tbp^{\Delta N/\Delta N}$ fetuses to maternal immune rejection suggests that recurrent immune-mediated spontaneous abortion in humans might be treated by controlling stochastic components, rather than the genetic components, of the condition. Using our mouse model system, one might consider testing whether altering prenatal conditions could decrease the penetrance of this triggering component, and thus increase survival of $tbp^{\Delta N/\Delta N}$ fetuses.

In summary, we have shown by genetic analyses of mice lacking the vertebrate-specific TBP N terminus that the first critical role for this acquired polypeptide in mammals is a $\beta 2m$ -dependent process that helps the fetus/placenta evade rejection by the mother's immune system. More generally, we posit that this domain can be viewed as a covalently linked TAF_{II} that coevolved with the adaptive immune system to allow regulated production of specific gene products by most cells of the organism. This, in turn, may potentiate the ability of the immune system to mount an effective and selective response to certain infectious agents.

Experimental Procedures

Production of Mice Bearing the $tbp^{\Delta N}$ Mutation, Mouse Lines, and Genotyping

Details of the targeting vector design and mouse production are presented in the legend to Figure 1 and in the Supplemental Data at <http://www.cell.com/cgi/content/full/110/1/43/DC1>. The mouse line involved in this study was produced in 1996 and, except where noted, data presented are on animals that have been backcrossed to C57Bl/6 for >7 generations. C57Bl/6, CD1, and SCID mice were from Charles River Laboratories. $Rag1$ and $\beta 2m$ knockout mice were from Jackson Labs. All animals were housed in sterile specialized care facilities. Animal care and all procedures involving live animals were approved by the MSU and/or UU institutional animal care and use committee(s) and followed established protocols (Hogan et al., 1994).

Genotypes of all mice and fetuses were determined by molecular analysis on tail or fetal DNA. The $tbp^{\Delta N}$ allele was genotyped as shown in Figure 1. For genotyping the $rag1^{-/-}$ and $\beta 2m^{-/-}$ alleles, we used internally controlled two-primer assays. Primers used for the $rag1$ allele were 5'-GCT CTA TCG TAA TTC TCA TGA CTG TG-3' and 5'-CAA GAG TGA CGG GCA CAG CCG GAG-3'. Primers used for the $\beta 2m$ allele were 5'-CTG AGC TCT GTT TTC GTC TG-3' and 5'-AAG TCC ACA CAG ATG GAG CGT-3'. The chromosomal locus for each mutant allele in this study is as follows: tbp , chr. 17; $scid$, chr. 16; $rag1$, chr. 2; and $\beta 2m$, chr. 2. As such, none of the other mutant alleles are linked to tbp ; however, rag and $\beta 2m$ are linked, and tbp is tightly linked to the MHC complex (<10 cMorgans).

Diploid/Tetraploid Chimeric Embryo Fusions

For diploid embryos, $tbp^{\Delta N/+}$ females were induced to super-ovulate and were mated with $tbp^{\Delta N/+}$ males. Morulae were harvested 2.5 days later and their zonae were removed (Hogan et al., 1994). Super-ovulated wild-type females were mated to wild-type males on the same schedule, and two-celled embryos were harvested at 1.5 d.p.f. The two-celled wild-type embryos were electro-fused to form one-celled tetraploid embryos using a BLS CF-150 impulse generator set at 100V and a square wave pulse of 25 μs in 0.3 M mannitol (Guillemot et al., 1994; Nagy et al., 1990). Tetraploid embryos were cultured overnight to form morulae, and their zonae were removed.

Diploid morulae were cocultured overnight with tetraploid morulae. The next day, blastocyst-stage chimeric embryos were implanted into pseudo-pregnant wild-type surrogates.

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